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Liver glycogen stores via ^{13}C magnetic resonance spectroscopy in healthy children: randomized, controlled study

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Running Title: Liver glycogen replenishment in children

Abbreviations:

AHP, Adiabatic half passage; AUC_{240min}, Area under the curve over 4 hours BIBD, Balanced incomplete block design; BMI, Body mass index; ^{13}C-MRS Carbon-13 magnetic resonance spectroscopy; i.netAUC_{240min}, Incremental net area under the curve over 4 hours; MR, Magnetic resonance; MRI, Magnetic resonance imaging;; PAL, Physical activity level; [LGly], Liver glycogen concentration; T1DM, Type I diabetes mellitus; TR, repetition time; 3T, 3 Tesla

Clinical Trial Registry number: NCT04278209 (www.clinicaltrials.gov)

Abstract

Background:

Owing to its role in glucose homeostasis, liver glycogen concentration ([LGly]) can be a marker of altered metabolism seen in disorders which impact health of children. However, there is a paucity of normative data for this measure in children to allow comparison with patients, and time-course assessment of [LGly] in response to feeding has not been reported. ^{13}C-magnetic resonance spectroscopy (^{13}C-MRS) is used extensively in research to non-invasively assess liver metabolites in adult health and disease, but similar measurements in children are lacking.

Objective:

The main objectives were to quantify the depletion of [LGly] after overnight fasting, and the subsequent response to feeding.

Design:

In a randomized, open-label, incomplete block design study, healthy, normal-weight children (8-12y) attended 2 evening visits, each separated by ≥5 days and directly followed by a morning visit. An individually tailored, standardized meal was consumed 3-hours prior to evening assessments. Participants then remained fasted until the morning visit. [LGly] was assessed once in the fed (20:00hrs) and fasted state (08:00hrs) using ^{13}C-MRS. After the 8:00hrs assessment, 200ml of a mixed-macronutrient drink containing 15.5g (402kJ) or 31g carbohydrate (804kJ), or water only, was consumed, with ^{13}C-MRS measurements then performed hourly for 4h. Each child was randomized to 2 of 3 drink options across the 2 mornings. Data are expressed as mean (SD).

Results:

Twenty-four children (13F:11M) completed the study (9.9(1.1)y, BMI percentile 45.7(25.9)). [LGly] decreased from 377.9(141.3) to 277.3(107.4) mmol·l^{-1} overnight; depletion rate 0.14(0.15) mmol·l^{-1}·min^{-1}. Incremental responses of [LGly] to test drinks differed (P<0.001),

with incremental net AUC of [LGly] over 4h ($i.netAUC_{240min}$) being higher for 15.5g (-67.1(205.8) $mmol \cdot l^{-1} \cdot 240min$; $P<0.01$) and 31g carbohydrate (101.6(180.9) $mmol \cdot l^{-1} \cdot 240min$; $P<0.005$) compared to water (-253.1(231.2) $mmol \cdot l^{-1} \cdot 240min$).

Conclusion:

After overnight fasting, [LGly] decreased by 22.9(25.1)%, and [LGly] $i.netAUC_{240min}$ was higher after subsequent consumption of 15.5g and 31g carbohydrate, compared to water.

Key words: muscle glycogen concentration, fasting, feeding, carbohydrate metabolism

Introduction

With the increased prevalence of obesity and metabolic disorders in the general population globally (1-3), there is a need for comprehensive understanding of the impact of diet and lifestyle on energy metabolism in humans across the lifespan. Whilst multiple studies have explored this in healthy and diseased adult cohorts, less work has focussed on children.

In adults, glycogen provides the primary acute-phase carbohydrate energy store and contributes to blood glucose concentration regulation between meals. Whilst muscle contains the largest reservoir of glycogen, liver glycogen metabolism contributes ~45% of total endogenous glucose production during the initial periods of fasting and thus plays a fundamental role in blood glucose homeostasis (4, 5). Liver glycogen content rapidly depletes with a period of fasting (6, 7) and is readily replenished in adults following consumption of carbohydrate. By contrast, fasting results in a limited reduction of the glycogen depots in adult skeletal muscle (7), with muscle switching its energy substrate metabolism predominantly to lipid oxidation during fasting (8) and insulin-stimulated muscle glucose uptake being reduced (9). However, similar information in children is lacking.

To our knowledge, only two studies have measured liver glycogen concentration ([LGly]) in children using Carbon-13 Magnetic Resonance Spectroscopy (^{13}C-MRS) previously (10, 11). One study (10) measured [LGly] following overnight fasting and 4-hours after two standardized meals (at breakfast and lunch), and reported [LGly] after the final meal was 36% above fasting values. In the other (11), daytime liver glycogen accumulation in children with type I diabetes mellitus (T1DM) was compared with that of controls without diabetes. Liver glycogen content was ascertained in the fasted (morning) and fed (afternoon) state and the ability of young children with T1DM to replace glycogen stores in the liver (depleted after an overnight fast) appeared to be comparable to control children. However, to what extent liver

and muscle glycogen concentrations are depleted in healthy children after overnight fasting and the temporal dynamics occurring after eating is unknown.

^{13}C-MRS is a well validated, non-invasive tool for organ specific glycogen measurement (12-14) and has been used in multiple adult studies over many years (15-17). The technique allows for safe and amenable sequential measurements of glycogen stores and is particularly suited to more vulnerable cohorts, such as children, but has not, to date, been widely exploited in this latter population.

The aim of the current study was to expand current knowledge of normative glycogen stores in children by using ^{13}C-MRS to assess the depletion of both liver and muscle glycogen after overnight fasting, and to investigate to what extent these stores are replenished with the intake of a small breakfast (equivalent to 1 or 2 servings of a chocolate malt beverage).

Subjects and Methods

Study design

A two-phase, randomized, controlled, open label, single center study was conducted to investigate the effect of consuming carbohydrate on liver and muscle glycogen stores in healthy children after overnight fasting. Specifically, the primary outcome measure was the net area under the concentration/time curve over 4 hours ($i.netAUC_{240min}$) for liver glycogen. The secondary outcome measures were the change in liver and muscle glycogen concentrations after an overnight (12-hour) fast, and the $i.netAUC_{240min}$ of the concentration/time curve for muscle glycogen. Although there was a theoretical potential for bias due to lack of blinding of participants to drink allocation, all analyses of magnetic resonance (MR) scans were carried out by individuals blinded to the intervention drink given. Furthermore, no subjective measures

were collected in participants that could have been impacted by them knowing the drink that they had consumed.

An interim analysis was carried out after the first phase had been completed to compute the number of additional subjects required to reach conditional statistical power. In phase 1 (9 participants; Aug 2020 to Dec 2020), a balanced incomplete crossover block design was employed to assess the effects of consuming 15.5g or 31g of available carbohydrate on liver glycogen concentration (compared to water; 6 participants per drink), with a second phase (15 participants; Apr 2021 to Nov 2021) using a balanced crossover design to measure the effects of consuming 15.5g of carbohydrate compared with water (15 participants per drink). The random allocation sequence for each phase was generated using an on-line system (iMedidata Rave; New York, USA) hosted by study sponsors (Société des Produits Nestlé SA) and individuals were randomized (using unstratified randomization) at the point of entering the study, with their participant number allocated sequentially. Enrollment was carried out independently by the research team at Nottingham. After a successful screening, participants attended the Sir Peter Mansfield Imaging Centre (University of Nottingham, UK) on 4 occasions; 2 evening visits, each separated by ≥5days and each directly followed by a morning visit.

Ethics

This study was conducted according to the Declaration of Helsinki 1973 guidelines (revised 2013) and all procedures involving human participants were approved by the University of Nottingham Medical School Ethics Committee (reference 426-1911). Written consent was obtained from all volunteers and their legal guardians, and the protocol was registered at www.clinicaltrials.gov (reference NCT04278209).

Participants

Healthy, normal weight, children at Tanner stages 1 or 2 (18, 19) and aged 8-12 years, were recruited from the local population through advertisement on social and traditional media platforms. Twenty-nine children who were interested in participating in the study and who fulfilled age and general health criteria attended the initial screening at the David Greenfield Human Physiology Unit (Medical School, Nottingham, UK) with their legal guardians. Body Mass Index (BMI) percentile was calculated from height and weight percentile using ethnicity-appropriate growth charts, with healthy BMI defined as being between the 5^{th} and 85^{th} percentile. Those with self-reported food allergies related to ingredients in the trial drinks, including lactose intolerance, were not recruited. See **Supplementary Material Figure 1** for Consort Diagram illustrating participant flow through the recruitment and experimental pathway.

Standard meal

Three hours prior to evening assessments (17:00hrs), an individually tailored meal (**Table 2**) (60% of energy as carbohydrate, 20% protein, 20% fat) providing 35% of daily energy requirement (determined as described below) was consumed by participants at home, with no further food intake from this meal until the following morning visit. Habitual diet and food preferences were previously determined from a 4-day dietary record (3 weekdays and 1 weekend day), with daily physical activity level (PAL) assessed over 5 days using a triaxial accelerometer (GT3X; ActiGraph LLC, Pensacola, FL, USA) worn at the waist. Both measurements were collected by participants after recruitment and returned to researchers before the first experimental visit. Dietary records were analyzed, and the standard evening meal designed, using a food composition database (Nutritics, Dublin, Ireland) (20), with the

activity data interrogated using manufacturer's software (Actilife V6; Actigraph LLC, Pensacola, FL, USA). The individual's PAL index was subsequently used as a multiplier for resting energy expenditure estimated from standard equations (21) to calculate daily energy requirement. Guardians were asked to record when their child ate the standard meal (start and end time) and if any of the meal was not consumed. Where the meal was not fully consumed, guardians were requested to photograph the food remaining and the actual intake (macronutrient and energy content) was estimated from these.

Study visits

On each evening visit (20:00hrs), participants underwent an approximately 1hr magnetic resonance (MR) scanning session (details below) at the Sir Peter Mansfield Imaging Centre, to assess volume of stomach contents, and liver and muscle glycogen concentration. They then returned the following morning (08:00hrs) having been instructed to have nothing to eat or drink, other than water, in the intervening period. Baseline MR measurements for stomach contents' volume and [LGly] were made before the consumption of a test drink according to the randomized drink allocation, with allocation for both sessions revealed after the first evening scan had been completed. Due to the extended fasting time required of the young participants when randomized to consuming water, the ~30-minute scan protocol to determine initial muscle glycogen concentration on all morning visits was scheduled immediately after drink ingestion to shorten the study day. Assessments of volume of stomach contents, liver and muscle glycogen concentration were then made every hour for 4 hours following drink consumption.

Interventional Products

The test drinks were 200ml of water or an equivalent volume of a mixed-macronutrient chocolate malt beverage (Milo®; Société des Produits Nestlé S.A.); the latter consisting of malt extract, skimmed milk powder, sugar, vegetable oil, and cocoa powder, which provided either 15.5g ('15.5g beverage') or 31g of carbohydrate ('31g beverage'); powder equivalent to 1 and 2 servings. Macronutrient and energy content of drinks are shown in **Supplementary Table 1**. A mixed macronutrient test drink was used in this study as it was a palatable means to provide the carbohydrate to these young participants and was expected to have a more rapid gastric emptying than an equivalent solid meal; reducing the post-drink data collection time and by extension the fasting time for children on the water visit. The chocolate malt beverages were supplied as powdered mixes, which were reconstituted with 200 ml of warm water immediately before consumption and were ingested within 10 minutes.

Magnetic Resonance Protocol

All MR measurements were acquired using a Philips 3T Achieva Magnetic Resonance Imaging (MRI) system (Philips, Best, Netherlands), with integrated proton body coil used for image acquisition. ^{13}C-MR spectra were obtained using a single-loop ^{13}C surface coil with integral butterfly proton decoupling channels (PulseTech, Surrey, UK). A ^{13}C-labelled urea sample was positioned in the center of the coil and used as a calibration reference signal.

At each timepoint, participants were placed in the scanner in the supine position and initial images acquired to determine gastric content volumes (~2 min). The ^{13}C surface coil was then placed over the liver (22) for acquisition of ^{13}C-MRS to determine [LGly] (~14 min). Finally, the ^{13}C surface coil was repositioned over the right thigh for acquisition of ^{13}C-MRS to determine muscle glycogen concentration (~26 min at the evening and initial morning scan, with subsequent assessments taking ~6 min).

219

220 *Volumes of Gastric Content* were measured using a fast gradient echo sequence (repetition time
221 = 2 ms, field of view = 300 x 200 x 300 mm, total scan time = 16 s, slice resolution = 120 x
222 192, 25 slices). Images were imported into Anlayze9 (Mayo Foundation, Minnesota, USA),
223 and the regions of interest were manually drawn around stomach contents to calculate volume
224 (23, 24).

225

226 *Liver Glycogen Concentrations* were measured using a non-localized short-repetition time
227 (TR) block pulse-acquire sequence (bandwidth = 7 kHz, repetition time = 280 ms, sample
228 points = 1024, number of averages = 3072, total scan time ~15 min) with pencil beam
229 shimming. Scout images were initially acquired to confirm correct coil positioning, followed
230 by a long-TR ^{13}C-reference scan for signal calibration (repetition time = 1500 ms, number of
231 averages = 20). The area under the glycogen doublets at ~101 ppm was determined by fitting
232 Gaussian curves using an in-house Matlab (MathWorks Inc, Massachusetts, USA) script and
233 scaling to the signal from the ^{13}C- Urea reference peak (~175 ppm). Absolute concentrations
234 were then calculated by comparison with a 200 mmol·l^{-1} glycogen phantom after correcting for
235 differences in transmit-receive field (B_1) sensitivity (23, 25, 26). Decrease in [LGly] from the
236 evening (fed) assessment to the next morning (fasted) assessment was summarized as a
237 percentage decrease, and between-visit coefficient of variation for fasting [LGly] was
238 computed. The overnight depletion rate of [LGly] over the fasting period was calculated across
239 all visits, with the depletion rate from initial morning to 240min assessment calculated at the
240 water visit. Incremental net area under the curve over the 4-hours (i.netAUC_{240min}) was
241 computed using the trapezoid method, accounting for areas both below and above the fasting
242 glycogen concentration.

243

Muscle glycogen concentrations were measured using a non-localized adiabatic half passage (AHP) hyperbolic-secant pulse-acquire sequence (bandwidth = 7 kHz, repetition time = 1358 ms, sample points = 512) with narrow-band proton decoupling (15) (AHP and decoupling used due to the variability and smaller size of muscle compared to liver in children). 1184 spectra were acquired and averaged at the evening and morning fasted time points (~26 min), whereas only 296 were averaged at all post-drink time points (~6 min) due to time constraints in the postprandial phase. The area under the single decoupled glycogen peak at ~101 ppm was determined by fitting a Gaussian curve using in-house Matlab script and scaling to the signal for the ^{13}C-Urea reference peak at (~175 ppm). Due to variations in muscle size affecting volumes within field of view, MR images were used to estimate participant specific B_1 sensitivity effects and correct the final signal. Absolute concentrations were then calculated by comparison with a 200 mmol·l^{-1} glycogen phantom (17).

Sample size

The initial sample size for the study was calculated for the primary objective, which was to determine the effects of consuming a mixed macronutrient drink, containing 15.5g or 31g of carbohydrate, on [LGly] after an overnight fast. The effects to be interrogated were the differences in [LGly] AUC_{240min} between each carbohydrate amount and water, with the purpose being to confirm the observed effect whilst controlling the experiment wise error rate at 0.05. In order to show a difference of 1.5 units within participant standard deviation in [LGly] i.netAUC_{240min} after consuming 31g of carbohydrate, with a power of 80% and an alpha-level (two-sided) of 0.05, it was estimated that 8 participants for each drink would be needed. This corresponded to a balanced incomplete block design (BIBD) with allocation of the sequences 0-1, 1-0, 1-2, 2-1, 0-2, 2-0 for 24 participants. An interim analysis was carried out after 9

participants had been completed to assess the conditional power of the primary variable, with a maximum of 24 participants retained.

Interim analysis

The stopping rule for success was according to Pocock; P<0.028 for [LGly] AUC_{240min} at interim and at final analysis (27). The randomization scheme of the BIBD was applied for 9 subjects and the purpose of the interim analysis was to allow any design changes based on conditional power. Interim analysis sample size assessment resulted in dropping the 31g beverage visit since significant differences with the water were already obtained at this stage despite the small number of participants in this group. The recruitment of a further 15 participants to undergo the water and 15.5g beverage visit was recommended in order to reach 67% conditional power.

Statistical Analysis

All data were coded and analyzed using SAS Life Science Analytical Framework version 5.2.3 (SAS Institute Inc., Cary, NC, USA). Data were initially checked for normality of distribution (using qq-plot and residuals vs. fitted values plot). Parametric data are described in the text and tables as the mean with standard deviation (SD) in parentheses. Normally distributed data in figures are the mean with error bars showing the standard error of the mean (SEM).

Glycogen overnight depletion was calculated as the average from all participants' overnight depletion measurements pooled across the two visits. Glycogen concentration AUC_{240min} assessments, made across the 3 drink options, were carried out using a linear mixed-effect model adjusting for drink, baseline [LGly] concentration values, BMI percentile, and the visit as fixed effects and participant as random effect. Gastric content evaluations, made across the

3 drink options, were carried out using a linear mixed-effect model adjusting for drink, baseline values, timepoint and the drink x time interaction as fixed effects and participant as random effect. The macronutrient and energy contents of the standard meals provided before evening visits were compared across visits using the linear mixed-effect model with drink as fixed effect and participant as random effect. Analysis was unpaired (due to incomplete crossover block design), used a two-tailed assessment and statistical significance was assumed where $P<0.028$ for [LGly] AUC_{240min} analysis and where $P<0.05$ for all other analyses. Post-hoc analysis was used to probe differences in repeated measures data.

Finally, associations between $i.netAUC_{240min}$ and carbohydrate content of the 3 drinks were investigated using correlation analysis and are expressed as Pearson's R.

Results

Participants

Twenty-four children (13F:11M) completed the study (9.9 (1.1) years, BMI percentile 45.7 (25.9)). In Phase 1 recruitment, 9 children (4F:5M) were randomized to 2 of the 3 drinks (31g beverage, 15.5g beverage or water) in the BIBD (n=6 per drink allocation), with 15 (9F:6M) being randomized in Phase 2 (after interim-analysis) to either the 15.5g beverage or water. Participant characteristics are shown in **Table 1**. No participant withdrew from the study after randomization.

Standard meal

The macronutrient and energy content of the standard meal provided before evening visits, and the actual amounts consumed are shown in **Table 2**. There were no differences in total energy or carbohydrate intake at the evening meal between drink allocation.

318

319 *Liver Glycogen Concentration*

320 Mean [LGly] decreased from 377.9 (141.3) to 277.3 (107.4) $mmol \cdot l^{-1}$ overnight (-22.9 (25.1)%;
321 equivalent to a depletion rate of 0.14 (0.15) $mmol \cdot l^{-1} \cdot min^{-1}$), with a between visit coefficient
322 of variation for fasting [LGly] being 21.5 (15.1)%. The impact of the test drink consumption
323 on [LGly] is shown in **Figure 1**. There was a difference in response of [LGly] between drinks
324 across the 4-hour post-ingestion period ($P<0.05$). Both the consumption of the 15.5g as well as
325 the 31g beverage showed a different response in [LGly] compared to water ($P<0.05$ and
326 $P<0.001$, respectively).

327

328 Similarly, there was a difference in 4-hour i.netAUC seen across the visits ($P<0.05$), with this
329 variable being significantly different after consumption of the 15.5g and 31g beverages
330 compared to water ($P<0.005$ and $P<0.01$, respectively). Moreover, exploratory analysis of the
331 relationship between the amount of carbohydrate consumed and $i.netAUC_{240min}$ showed a linear
332 carbohydrate 'dose' response in $i.netAUC_{240min}$ ($R=0.51$; $P<0.001$; **Figure 2**). At 2 hours after
333 test drink consumption, mean liver glycogen repletion (from fasting) was 5.8 (29.6)%
334 following consumption of the 15g beverage and 34.6 (57.0)% after the 31g beverage, whereas
335 following water consumption, [LGly] decreased by 21.2 (29.4)% during the first 2 hours after
336 intake ($P<0.005$). However, due to the small sample size in the 31g group, inferences made for
337 this group should be interpreted with caution.

338

339 *Muscle Glycogen*

340 Muscle glycogen concentrations in the fed (evening) and fasted (morning) state were not
341 different; 114.9 (38.2) and 116.3 (40.4) $mmol \cdot l^{-1}$, respectively. Moreover, there was no
342 difference in the response of muscle glycogen concentrations, or 4-hour' i.netAUC between

the 15.5g beverage and water visit. Technical difficulties resulted in a complete data set for only 1 participant at the 31g beverage visit for muscle glycogen concentration and thus the differences between this test drink and water could not be compared.

Gastric Emptying

Because gastric emptying will influence the uptake of carbohydrates into the circulation, and thus [LGly] replenishment, we also measured the volume of gastric contents over the 4-hour postprandial timepoints (**Figure 3**). At the 1hr assessment, water appeared to have completely emptied from the stomach, with mean gastric content volumes reaching fasted volumes by 2hrs and 3hrs after consumption of the test drinks containing 15.5g and 31g carbohydrate, respectively. Gastric emptying half-life was estimated as 32.4 (0.5) min for water, 62.5 (6.1) min for 15.5g and 88.3 (8.4) min for 31g beverages ($P<0.001$).

There was a difference in response of mean volume of gastric contents between drinks across the 4-hour post-ingestion period with the Time x Drink interaction being statistically significant ($P<0.001$). Both the consumption of the 15.5g as well as the 31g beverage showed a different response in volume of gastric contents compared to water at the timepoint of 60 minutes after drink consumption ($P<0.001$ for both comparisons). At remaining timepoints, comparisons between drinks did not reach significance.

Discussion

The current study expands knowledge of normative glycogen stores in children by using ^{13}C-MRS to assess the impact of overnight fasting on both liver and muscle glycogen. Moreover, the time course response and extent to which these stores were replenished with intake of 15.5g and 31g available carbohydrate (equivalent to 1 or 2 servings of a chocolate malt beverage) are presented. To our best knowledge, it is the first time that these have been quantified in children.

Liver glycogen concentration, depletion and repletion could be important markers of altered metabolism seen in disorders which impact the health of children due to the liver's role in maintaining glucose homeostasis. Biopsies would traditionally be taken to assess liver glycogen content but concerns around the safety of this invasive technique limits its use in healthy children, and makes it unsuitable for sequential measurements to assess depletion or repletion rates in any child. ^{13}C-MRS has been used extensively in research to non-invasively assess liver metabolites in adult health and disease. However, this technique has not been widely exploited in pediatric cohorts. Consequently, there is a paucity of normative data for [LGly] in children to allow comparison with patient cohorts. Indeed, we are aware of only 2 such reports in the literature (10, 11). In the present study, the MR scanning protocols were well tolerated by the young participants, making it an acceptable technique to use in this age group.

Our data indicated that [LGly] decreased by approximately 23% overnight. In adults, [LGly] has been reported to decrease by ~0.2 $mmol \cdot l^{-1} \cdot min^{-1}$ in the first 22 hours of fasting, a rate that was near constant over this time frame (5). In the current study, this depletion rate in children over the 12-hour overnight fast was lower; at 0.14 $mmol \cdot l^{-1} \cdot min^{-1}$, although it has been hypothesized that depletion rates could be greater than seen in adults (28). The pre-fasting [LGly] in the current protocol was assessed 3-hours after the standard evening meal and at this timepoint the stomach still showed evidence of the meal being present. As participants had not fully digested and absorbed the meal at this 'fed' assessment, it is possible that [LGly] may not have reached the postprandial 'peak' 3 hours after the meal and therefore could be underestimated. Consequently, the liver glycogen depletion rate in children over the 12-hour fast may be higher than calculated by the current data. Indeed, on the 'water' study day, the reduction of [LGly] with continued fasting over the morning (which according to adult data would continue the same depletion rate as seen overnight), showed a higher 'morning'

depletion rate (0.38 (0.30) $mmol \cdot l^{-1} \cdot min^{-1}$) than observed overnight. Logistical considerations around meal and bedtimes of this age group (and the duration that the young participants remained fasted during 'water' visits) meant that scheduling a later evening visit or an earlier evening meal was not possible. Future studies would benefit from using a standard pre-fast meal which has a faster gastric emptying and absorption time.

The fasting [LGly] of children in the current cohort was 277 $mmol \cdot l^{-1}$. A previous study using ^{13}C-MRS showed that fasting [LGly] in healthy children aged 6 to 12 years, was lower (median [range] 178 [120-203] mM glycosyl units) (11). However, before the overnight fast in this study, no standardized meal was provided (and information about the composition and size of prior meals was not described), whereas the current protocol provided a high carbohydrate meal (35% of total daily energy requirement; 60% of energy as carbohydrate) which may have contributed to the higher [LGly] seen the following morning. Due to the limited information available in the literature, it is difficult to determine whether fasting values obtained in the current study were higher than would be expected.

Breakfast is considered an important meal of the day, and it is recommended that breakfast should contribute ~20% of a child's daily energy requirement, with 60% of energy being provided by carbohydrate (29). Research has shown that breakfast consumption improves diet quality, and there may be functional benefits associated with breakfast eating in children (30-32). Consumption of the 15.5g test drink after the overnight fast (equivalent to ~0.5g of available carbohydrate/ kg body weight) maintained fasting [LGly] for 2 hours and delayed the decrease seen with continued fasting, whereas consumption of the 31g test drink led to a 35% increase (from fasting levels) in [LGly] at the 2-hour timepoint, which decreased to overnight fasting concentrations over the subsequent 2 hours. A standard serving-size equivalent of breakfast cereal and milk, or a slice of bread and jam, provide ~25-32g of carbohydrate, which

417 approximately equates to the 31g beverage in the current study. However, in the UK it is
418 estimated that the average intake of carbohydrate at breakfast in 5-12-year-olds is
419 approximately double this amount (32). Inferences from the linear 'dose' response obtained in
420 the current study, would suggest that this higher carbohydrate intake would result in a further
421 increased net.iAUC$_{240min}$ for [LGly]. The implications of delaying glycogen depletion or
422 increasing glycogen concentrations in the liver on function (e.g. cognitive, physical
423 performance) in children was not measured in this study and existing evidence around the
424 benefits of breakfast eating on cognitive function is ambiguous (30). However, the relationship
425 between functional measures and carbohydrate stores, plus the impact of breakfast
426 macronutrient composition, glycemic load, or glycemic index on those stores is warranted.
427 These future studies would be feasible in a young cohort using MR methods.

428 The glycogen concentration of the children's muscle was lower in the fasted state than
429 previously observed in adults in our laboratory (33), yet higher than reported by others (34,
430 35). Although data are scarce, muscle glycogen content, determined in biopsies, suggests that
431 this variable in 11-13-year old males may be lower than reported in adults (36-38). There were
432 a number of technical difficulties in acquiring muscle glycogen measurements in the current
433 study due to the B_1 field inhomogeneities and the variability in size and shape of the quadriceps
434 muscles. In order to overcome these challenges, a Biot-savart static field approximation was
435 used to estimate total change compared to a standard phantom based on acquired images. In
436 addition, the signal from muscle was much smaller than the liver due to the distribution of
437 glycogen through all musculoskeletal tissue, resulting in a low signal to noise ratio. This was
438 addressed by acquiring the signal using adiabatic pulses (39) and decoupling (40), and by using
439 a longer muscle scan time at the evening 'fed' and morning 'fasted' scans; the latter increasing
440 signal to noise ratio at these timepoints. Scheduling restrictions did not allow the extended scan
441 time at postprandial timepoints and as a consequence the variability in these data was greater.

Future studies investigating muscle glycogen in children will need to consider the impact of these factors on imaging time and analysis. However, the absence of a change in muscle glycogen levels detected in the current study following overnight fasting (using the extended acquisition time), or with intake of carbohydrate, is similar to what has previously been observed in adults (41).

In conclusion, liver glycogen concentration decreased by 23% in healthy children (8-12 years) after an overnight fast. Subsequent consumption of 15.5g of available carbohydrate, maintained liver glycogen concentration at overnight fasting levels for 2 hours and delayed the further decrease seen after water intake. At this 2-hour timepoint, liver glycogen concentration was 35% higher than fasting values after ingestion of 31g of carbohydrate, with this measure staying above fasting concentrations for 4 hours after consumption. The 4-hour i.netAUC for liver glycogen concentration was higher after consumption of 15.5g and 31g carbohydrate compared to water. Muscle glycogen concentration at rest did not change significantly with fasting or refeeding. Results from this study expand the current limited knowledge of normative glycogen stores in children.

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469 Author disclosures - AMHH, AG, LE, NR, KM, DB, and IAM were employees of Société

470 des Produits Nestlé SA. SJB, AS, PG and EJS report no conflicts of interest.

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Table 1: Participant baseline characteristics according to each drink option.

	water (n=21)	**15.5 g beverage (n=21)**	**31 g beverage (n=6)**
Age (y)	9.8 (1.1)	10.0 (1.2)	10.0 (1.4)
BMI[1] (%)	43.2 (26.6)	42.8 (25.4)	64.0 (17.0)
Female (n)	13	12	1

Values are the mean with standard deviation in parentheses .[1]BMI, Body Mass Index

Table 2: Energy and macronutrients content of the prescribed standardized evening meal presented to, and consumed by the participants, according to drink allocation (water, 15.5g beverage and 31g beverage) the following morning.

	Meal prescribed			
	Energy (kJ)	**Proteins (g)**	**Carbohydrates (g)**	**Fat (g)**
water (n=21)	2732 (360)	33 (5)	97 (13)	15 (2)
15.5g beverage (n=21)	2699 (347)	32 (4)	95 (14)	15 (2)
31g beverage (n=6)	2812 (346)	32 (6)	99 (15)	17 (2)
	Meal consumed			
	Energy (kJ)	**Proteins (g)**	**Carbohydrates (g)**	**Fat (g)**
water (n=21)	2657 (448)	32 (6)	94 (17)	15 (2)
15.5g beverage (n=21)	2594 (473)	31 (6)	91 (18)	15 (3)
31g beverage (n=6)	2774 (351)	32 (6)	97 (14)	17 (2)

Data are the mean with standard deviation in parentheses.

Legends for figures

Figure 1: incremental change in liver glycogen concentrations from fasted morning state, measured hourly for 4 hours after the drink. Data are the mean with error bars indicating SEM.  Black circles: water (n=6);  Black squares: drink containing 15.5 g of carbohydrate (n=21);  Grey triangles: drink containing 31 g of carbohydrate (n=21). Drink effect: $P<0.05$. $P<0.05$ for 15.5 g beverage vs. water and $P<0.001$ for 31 g beverage vs. water.

Figure 2: Incremental net Area Under the Curve (i.netAUC) for liver glycogen concentrations over the 4h-postprandial period for each of the 3 drinks (n=21, 21 and 6 for respectively water, 15.5 g and 31 g beverage). The figure shows the individual values, with boxes indicating the 25^{th} and 75^{th} percentile, and error bars showing the data range. The bold lines within the boxes indicate the median, with crosses showing the group mean. 15.5 g beverage: drink containing 15.5g of carbohydrate; 31 g beverage: drink containing 31 g of carbohydrate. * $P<0.05$ compared to water.

Figure 3: Incremental change in gastric content volume compared to fasted morning state, measured hourly for 4 hours after the drink. Data are the mean with error bars indicating SEM.  Black circles: water (n=6);  Black squares: drink containing 15.5 g of carbohydrate (n=21);  Grey triangles: drink containing 31 g of carbohydrate (n=21). Time x Drink: $P<0.001$. At t=60 min, $P<0.001$ for 15.5 g beverage vs. water and $P<0.001$ for 31 g beverage vs. water.